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General and Comparative Endocrinology 137 (2004) 312–321
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Communications in Genomics and Proteomics

Molecular cloning of proopiomelanocortin cDNA and multi-tissue mRNA expression in channel catfish

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Received 18 November 2003; revised 17 March 2004; accepted 22 March 2004
Available online 17 April 2004

Abstract

Channel catfish (*Ictalurus punctatus*) proopiomelanocortin (*POMC*) cDNA was cloned to investigate its structure, evolution, and expression in different tissues. *POMC* is an important gene in the hypothalamus–pituitary–adrenal axis, the main mediator of the stress response. *POMC* gene was isolated from a pituitary cDNA library and nucleotide sequence was determined. *POMC* cDNA is composed of 1164 nucleotides with a 639 nucleotide open reading frame encoding a protein of 212 amino acids. Catfish POMC protein contains a signal peptide (SP, Met^{1}–Ala^{28}), N-terminal peptide (Gln^{29}–Glu^{101}), adrenocorticotropic hormone (ACTH, Ser^{104}–Met^{142}), α-melanocyte stimulating hormone (α-MSH, Ser^{104}–Val^{116}), corticotropin-like intermediate lobe peptide (CLIP, Arg^{121}–Met^{142}), β-lipotropin (β-LPH, Glu^{145}–His^{212}), γ-lipotropin (γ-LPH, Glu^{145}– Ser^{177}), β-MSH (Asp^{161}–Ser^{177}), and β-endorphin (β-EP, Tyr^{180}–His^{212}). Catfish POMC protein does not contain a γ-MSH region and most of the joining peptide and part of the γ-LPH are deleted. Protein sequence alignment showed the highest similarity with the carp (*Cyprinus carpio*) POMC I (66.5%) and POMC II (67%), while the sea lamprey (*Petromyzon marinus*) POC (17.9%) and POM (18.8%) were the most divergent. The average similarity was 46.95% among the 44 POMC proteins from 36 different species analyzed. Compared to the *POMC* mRNA levels in the pituitary, the concentration of the *POMC* mRNA was 0.0594% in the anterior kidney and 0.0012–0.0045% in all the other tissues except in the skin where the lowest expression (0.0005%) was observed. Overall architecture of channel catfish POMC is highly similar to those from other teleosts.

Keywords: Fish; Pituitary; ACTH; MSH; β-Endorphin; Stress

1. Introduction

The vertebrate endocrine system is activated in response to external and/or internal changes sensed as stressful. These changes are detected by the central nervous system and immune system, and responded to by the main mediator hypothalamic–pituitary–adrenal axis (HPA) in which proopiomelanocortin (POMC) derived peptides play an important role (Slominski et al., 2000). In the pituitary of mammalian species, *POMC* is mainly expressed in the corticotrope cells of the pars distalis and melanotrope cells of the pars intermedia. Lobe-specific processing and post-translational modifications of the precursor protein yield biologically active peptides including adrenocorticotropic hormone (ACTH), melanocyte stimulating hormones (MSH), and β-endorphin (β-EP, Castro and Morrison, 1997; Smith and Funder, 1988).

In general, mammals transcribe only one *POMC* gene (Drouin et al., 1989) although two non-allelic forms of *POMC* gene have been detected in some fish species. Agnathan lamprey (*P. marinus*) ACTH and MSHs are encoded separately by two distinct *POMC*s, proopiocortin (*POC*) and proopiomelanotropin (*POM*, Heinig et al., 1995; Takahashi et al., 1995). Duplicated

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doi:10.1016/j.ygcen.2004.03.012

copies of *POMC* exist in chondrostean Mississippi paddlefish (*Polyodon spathula*) and sturgeon (*Acipenser transmontanus*) and in teleost trout (*Oncorhynchus mykiss*), salmon (*Oncorhynchus keta*), and carp while only a single copy was found in sarcopterygian lungfish (*Protopterus annectens* and *Neoceratodus forsteri*) and teleost zebrafish (Alrubaian et al., 1999; Amemiya et al., 1999a; Arends et al., 1998; Danielson et al., 1999; Hansen et al., 2003; Okuta et al., 1996; Salbert et al., 1992, see Table 1 for accession numbers). In tetrapods, DNA domain duplication resulted in three MSH coding regions named as γ-MSH, α-MSH, and β-MSH (Nakanishi et al., 1979). The number of MSH sequences changes in different species of fish. For example, non-teleost lungfish, paddlefish, sturgeon, bichir (*Polypterus senegalus*), and gar (*Lepisosteus osseus*) contain a γ-MSH region while it is deleted in teleost fish such as tuna (*Thunnus obesus*), tilapia, trout, zebrafish, eel (*Anguilla rostrata*), salmon, and carp (see Table 1 for accession numbers). In addition to these three MSHs, a new δ-MSH has been identified in the chondrichthyan dogfish (*Squalus acanthias*) and stingray (*Dasyatis akajei*, Amemiya et al., 1999b, 2000). Similar to teleosts, neither of the agnathan lamprey *POC* and *POM* genes encodes γ-MSH and δ-MSH. These are indicators of early appearance of γ-MSH in the evolution of gnathostomes and the evolution of *POMC* by duplication, insertion, deletion, and subsequent mutations of

Table 1
POMC sequences from vertebrates and their similarity to channel catfish POMC

Species	Database	Accession No.	Amino acid No.	Percent identity
Carp II (*Cyprinus carpio*)	NCBI	CAA74967	222	67.0
Carp I (*Cyprinus carpio*)	NCBI	CAA74968	222	66.5
Zebrafish (*Danio rerio*)	NCBI	AAM93491	222	64.2
Longnose gar (*Lepisosteus osseus*)	NCBI	AAB03227	259	58.0
Trout A (*Oncorhynchus mykiss*)	NCBI	CAA49466	253	57.5
Mississippi paddlefish A (*Polyodon spathula*)	NCBI	AAD41263	264	55.7
Sturgeon A (*Acipenser transmontanus*)	NCBI	AAD55816	264	55.6
Mississippi paddlefish B (*Polyodon spathula*)	NCBI	AAD41264	264	54.2
American eel (*Anguilla rostrata*)	NCBI	AAF22344	216	53.8
Sturgeon B (*Acipenser transmontanus*)	NCBI	AAD17806	264	52.8
Tilapia (*Oreochromis mossambicus*)	NCBI	AAD41261	208	51.4
Tuna (*Thunnus obesus*)	NCBI	BAA35125	222	51.4
Yellowfin seabream (*Acanthopagrus latus*)	NCBI	AAF22342	232	50.9
Sobaity seabream (*Sparidentex hasta*)	NCBI	AAF22343	223	49.5
Australian lungfish (*Neoceratodus forsteri*)	NCBI	AAD37347	255	49.1
Barfin flounder B (*Verasper moseri*)	NCBI	BAB18468	214	49.1
Halibut I (*Paralichthys olivaceus*)	NCBI	AAG16978	216	49.1
Senegal bichir (*Polypterus senegalus*)	NCBI	AAL73510	259	48.6
African lungfish (*Protopterus annectens*)	NCBI	AAD29144	255	48.1
Xenopus A (*Xenopus laevis*)	NCBI	CAA42013	259	46.7
Marsh frog (*Rana ridibunda*)	SWISS-PROT	P22923	260	46.2
Mud turtle (*Pelodiscus sinensis*)	NCBI	AAM34798	261	45.3
Xenopus B (*Xenopus laevis*)	NCBI	CAA42012	260	45.3
Halibut II (*Paralichthys olivaceus*)	NCBI	AAG28378	194	44.8
Giant toad (*Bufo marinus*)	NCBI	AAF06345	259	44.3
Guinea pig (*Cavia porcellus*)	SWISS-PROT	P19402	256	44.3
Barfin flounder A (*Verasper moseri*)	NCBI	BAB18467	199	44.2
Chicken (*Gallus gallus*)	NCBI	BAA34366	251	43.9
Cow (*Bos taurus*)	SWISS-PROT	P01190	265	43.9
Spadefoot toad (*Spea multiplicata*)	NCBI	AAD21040	258	43.9
Bull frog (*Rana catesbeiana*)	SWISS-PROT	P11885	263	43.4
Pig (*Sus scrofa*)	NCBI	CAA27248	267	43.4
Rat (*Rattus norvegicus*)	SWISS-PROT	P01194	235	43.4
Spiny dogfish (*Squalus acanthias*)	NCBI	BAA32606	320	43.4
Human (*Homo sapiens*)	SWISS-PROT	P01189	267	42.9
Monkey (*Macaca nemestrina*)	NCBI	AAA66022	264	42.9
Pig-tailed macaque (*Macaca nemestrina*)	SWISS-PROT	P01201	264	42.9
Mouse (*Mus musculus*)	SWISS-PROT	P01193	235	42.5
Chum salmon II (*Oncorhynchus keta*)	SWISS-PROT	P10000	226	40.6
Short-tailed opossum (*Monodelphis domestica*)	NCBI	AAL13338	272	40.1
Trout B (*Oncorhynchus mykiss*)	NCBI	CAA49467	240	40.1
Red stingray (*Dasyatis akajei*)	NCBI	BAA35126	304	38.2
Sea lamprey POM (*Petromyzon marinus*)	NCBI	BAA09492	245	18.8
Sea lamprey POC (*Petromyzon marinus*)	NCBI	BAA09491	278	17.9

the γ-MSH region (Amemiya et al., 1997, 2000). In contrast to MSH variation, one β-EP is consistently present at the C-terminal of the POMC protein. Three highly conserved regions of POMC, N-terminal, ACTH, and β-EP, are suitable for aligning sequences while moderately variable γ-MSH and β-MSH regions and highly variable joining peptide (JP) and the N-terminal region of γ-lipotropin (γ-LPH) are suitable for delineating phylogenetic relationships (Alrubaian et al., 2002; Graybeal, 1994).

The *POMC* gene is predominantly expressed in the pituitary gland where it constitutes about one-third of all mRNA (Inoue et al., 1979; Pawelek, 1993). *POMC* mRNA and POMC derived peptides have been detected in most tissues including the immune system (Slominski et al., 2000). Presence of POMC derived peptides has been reported in different fish species by immunohistochemistry and by radioimmunoassay (Dores et al., 1990, 1994; Joss et al., 1990; Keller et al., 1994; Masini et al., 1999; Olivereau and Olivereau, 1990; Vallarino et al., 1992). Similarly, ACTH expression has been identified in channel catfish (*Ictalurus punctatus*) leukocytes by radioimmunoassay (Arnold and Rice, 2000). High-level expression of carp (*C. carpio*) *POMC I* and *POMC II* mRNAs has been detected in the pituitary while their expression levels were low in the brain (Arends et al., 1998). Quantitative expression of sea bass (*Dicentrarchus labrax*) *POMC* in pituitary, liver, gonad, and anterior kidney has recently been reported (Varsamos et al., 2003). Spatial distribution and developmental mRNA expression of *POMC* in zebrafish (*Danio rerio*) embryos have been reported (Hansen et al., 2003). Despite recent studies, little is known about *POMC* gene expression in non-pituitary tissues of fish species.

This study focuses on *POMC* in channel catfish, a member of family Ictaluridae under the order of Siluriformes (Nelson, 1994). Channel catfish, an important aquaculture species in the United States, are exposed to various and numerous stressors during pond culture that affect production. Determining the catfish *POMC* sequence and its expression is the initial step toward future studies addressing the role of POMC derived peptides in the stress response of catfish.

2. Materials and methods

2.1. Experimental fish

Fish for the construction of the pituitary complementary DNA (cDNA) library were previously defined (Karsi et al., 1998). For expression analysis, 18 month-old fish (104.0 ± 3.6 g, 26.2 ± 1.1 cm) obtained from the USDA-ARS, Catfish Genetics Research Unit at Stoneville, MS were acclimated in 150-L tanks for two weeks and fed a commercial diet. External stress was minimized during the acclimation of the fish and the collection of fish samples. Following accepted standards of animal care, approved by the Institutional Animal Care and Use Committee (IACUC) according to USDA-ARS policies and procedures, 10 fish were rapidly killed by a high dose of 8 mg/L metomidate plus 200 mg/L MS-222 and the pituitary, brain, gill, heart, anterior kidney, posterior kidney, stomach, liver, spleen, muscle, and skin tissues were immediately collected into RNAlater Tissue Collection:RNA Stabilization Solution (Ambion, Austin, TX) and stored at −20 °C overnight.

2.2. Sequencing and sequence analysis of POMC cDNA

Identified cDNA clones were grown in 2-ml LB medium overnight and plasmid DNA was prepared using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The sequences of plasmid inserts were determined using a CycleSeq-farOUTe DNA sequencing kit (Display Systems Biotech, Vista, CA) and M13 forward and reverse primers. Sequencing reactions were performed in a PTC-100 thermal cycler (MJ Research, Waltham, MA). The cycling conditions were as follows: initial denaturation at 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 45 s. Sequences were analyzed on an automatic Long ReadIR 4200 DNA sequencer (LI-COR Biosciences, Lincoln, NE). Two approaches were taken for the confirmation of the full-length mRNA sequence. First, sequences produced in this study and previously reported partial EST sequences (Karsi et al., 1998) were used to determine the consensus sequence by aligning them using MegAlign program of DNASTAR software (DNASTAR, Madison, WI). Discrepancies were rechecked on sequencing gel images and cDNAs were resequenced when necessary. Second, an independent sequence confirmation was done on identified *POMC* ESTs from the channel catfish brain cDNA library at USDA-ARS in Stoneville, MS. Briefly, dry-stored *POMC* plasmids were rehydrated in ddH_2O and Z-Competent *Escherichia coli* cells (Zymo Research, Orange, CA) were transformed and plated on LB-agar plates containing 50 μg/ml ampicillin. Plasmid DNA was prepared as indicated above. Big Dye 3 Cycle Sequencing Kit v3.0 (Applied Biosystems, Foster City, CA), M13 forward and reverse primers, and *POMC* specific forward (5′-GTTTCATGAAGTCTTG GGATGAG-3′) and reverse (5′-ATGGTGAAAGTA CTGCTGGCTAC-3′) primers were used in the sequencing reactions performed in a PTC-200 thermal cycler (MJ Research, Waltham, MA). The cycling conditions were as follows: initial denaturation at 96 °C for 2 min followed by 24 cycles of 96 °C for 30 s, 50 °C for 1 min, and 60 °C for 4 min. Sequence information was obtained using an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) and the analysis mentioned above was repeated.

2.3. Structural analysis of catfish POMC protein

The catfish *POMC* mRNA sequence was entered into the EditSeq program of the DNASTAR software package and translated into amino acid sequences using standard genetic code. POMC protein sequences of various species were retrieved from NCBI and SWISS-PROT databases and entered into the EditSeq program. Multiple protein sequence alignments were performed using the CLUSTAL method (Higgins and Sharp, 1989) of the MegAlign program of the DNASTAR software package. Detailed analysis of this alignment revealed the structure of the catfish POMC protein in addition to sequence distances. Post-translational modification patterns were determined using ScanProsite (http://us.expasy.org/tools/scanprosite/).

2.4. Total RNA extraction and cDNA synthesis

Total RNAs from the collected tissues were isolated using RNAwiz RNA Isolation Reagent (Ambion, Austin, TX). The tissues were immediately transferred into RNase-free tubes containing 10 volumes of RNAwiz reagent and were disrupted by an electric homogenizer. The homogenates were incubated at room temperature (RT) for 5 min and then 0.2 ml of starting volume chloroform was added to each sample. The samples were mixed and incubated at RT for 10 min followed by a centrifugation at 4000 rpm for 30 min using a 5810R centrifuge (Brinkmann, Westbury, NY). After centrifugation, the upper aqueous phase was transferred into a clean tube and 0.5 starting volume of RNase-free H_2O was added. The samples were mixed well and one starting volume of isopropanol was added to precipitate the RNA. Precipitated RNA pellet was washed in 1.5 starting volume of cold 75% ethanol, centrifuged, and air-dried. Finally, the RNA pellets were dissolved in 50 μl nuclease-free H_2O, quantified, and stored at −80 °C. Five micrograms of the total RNA was treated with a DNA-free DNAse Treatment and Removal Kit (Ambion, Austin, TX) to remove contaminating DNA. Following the DNA-free treatment, 750 ng of total RNA was converted to cDNA using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA) and cDNA quality was confirmed prior to expression analysis by amplifying different genes of various sizes including glyceraldehyde-3-phosphate dehydrogenase, 18S ribosomal RNA (*18S rRNA*) gene, follistatin, and *POMC*.

2.5. Real-time PCR analysis

Tissue expression of the *POMC* gene was detected by real-time PCR. Primers and hybridization probes were designed using Primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and synthesized commercially (Integrated DNA Technologies, Coralville, IA). Introns/exon junctions were predicted by alignment of catfish *POMC* mRNA sequences with available *Xenopus POMC A* (*Xenopus laevis*, Accession No. X59370) and chicken (*Gallus gallus*, Accession No. AB019555) genomic sequences. The forward primer (5′-TGACAGAAATATCCTGGAATGC-3′) included 16 bases from exon 2 and 6 bases from exon 3 for cDNA specific amplification. The probe sequence contained a 5′ FAM fluorophore and 3′ BHQ quencher. Real-time PCR analysis was performed using the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Amplification of *POMC* and control *18S rRNA* were checked to determine any competition between primers prior to expression analysis. Having confirmed that both primer pairs did not inhibit each other, tissue expressions were conducted in a 25 μl PCR reaction containing 5 μl of cDNA converted from 2.5 ng of total RNA, 200 nM *POMC* probe, 400 μM *POMC* forward and reverse (5′-TCGTGATTTCACTGGGTATGAG-3′) primers, 100 μM *18S rRNA* probe, 100 μM *18S rRNA* forward (5′-GAGAAACGGCTACCACATCC-3′) and reverse (5′-GATACGCTCATTCCGATTACAG-3′) primers, and 12.5 μl of 2× iQ Supermix (Bio-Rad, Hercules, CA). The 50 cycles of the two-step PCR program consisted of 3 min of initial polymerase activation at 95 °C followed by 15 s of denaturation at 95 °C and 45 s of annealing/extension at 60 °C in which fluorescent signal was detected. Each set of samples were run three times and each plate contained duplicate cDNA standards in tenfold serial dilutions, quadruplicate unknown cDNA samples, and negative controls. PCRs were loaded onto 2% agarose gel containing 0.5 μg/ml ethidium bromide to confirm amplification and size of the PCR products.

2.6. Relative quantification of POMC expression

The quantities of *POMC* message from different tissues were normalized with *18S rRNA* to compensate for variations in input RNA amounts. Relative levels of expression were determined according to user bulletin #2 (Applied Biosystems, Foster City, CA). Total RNA isolated from the catfish pituitary was quantified using a computer-controlled double-beam Lambda EZ210 spectrometer (Perkin–Elmer, Boston, MA) and serial dilutions of cDNA converted from known amounts of the total RNA sample were used to construct the standard curve for *POMC* and *18S rRNA*. For each of the unknown tissue samples, *POMC* and *18S rRNA* amounts were calculated by iCycler iQ Real-Time PCR Detection System Software v 3.0a (Bio-Rad, Hercules, CA) using linear regression analysis from their respective standard curves. Normalization was carried out by dividing the average value of *POMC* by the average value of *18S rRNA* in each tissue. Relative *POMC* expression was determined by dividing the normalized value of *POMC* in each tissue by the normalized value of *POMC* in the skin.

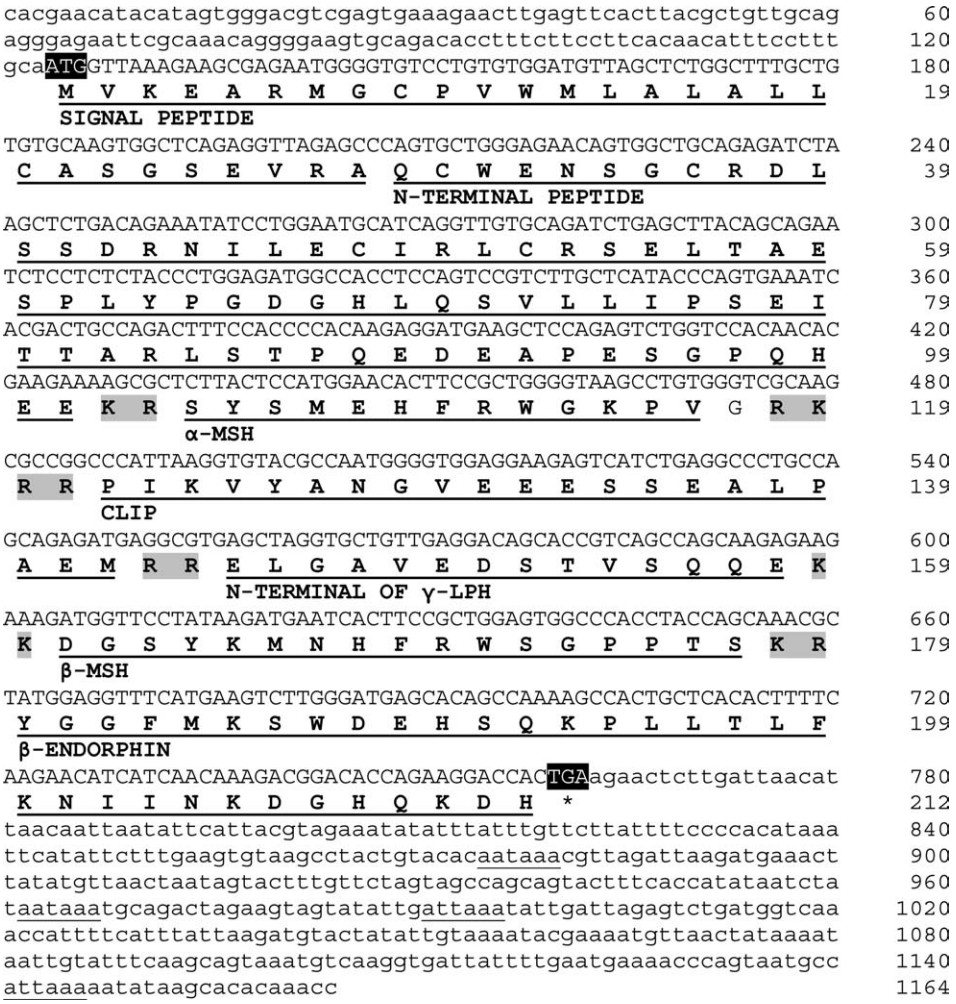


Fig. 1. Nucleotide and deduced amino acid sequences of cDNA encoding channel catfish (*I. punctatus*) *POMC*. Numbers on the right indicate nucleotide and amino acid sequences. Coding regions are in uppercase letters and non-coding regions are in lowercase letters. The start codon (ATG) and stop codon (TGA) are indicated in white letters on the black background. Two common (AATAAA) and two potential (ATTAAA) polyadenylation signal sequences are underlined. The dibasic amino acids, potential cleavage sites for processing enzymes, are shaded. POMC derived peptides are underlined and labeled.

3. Results

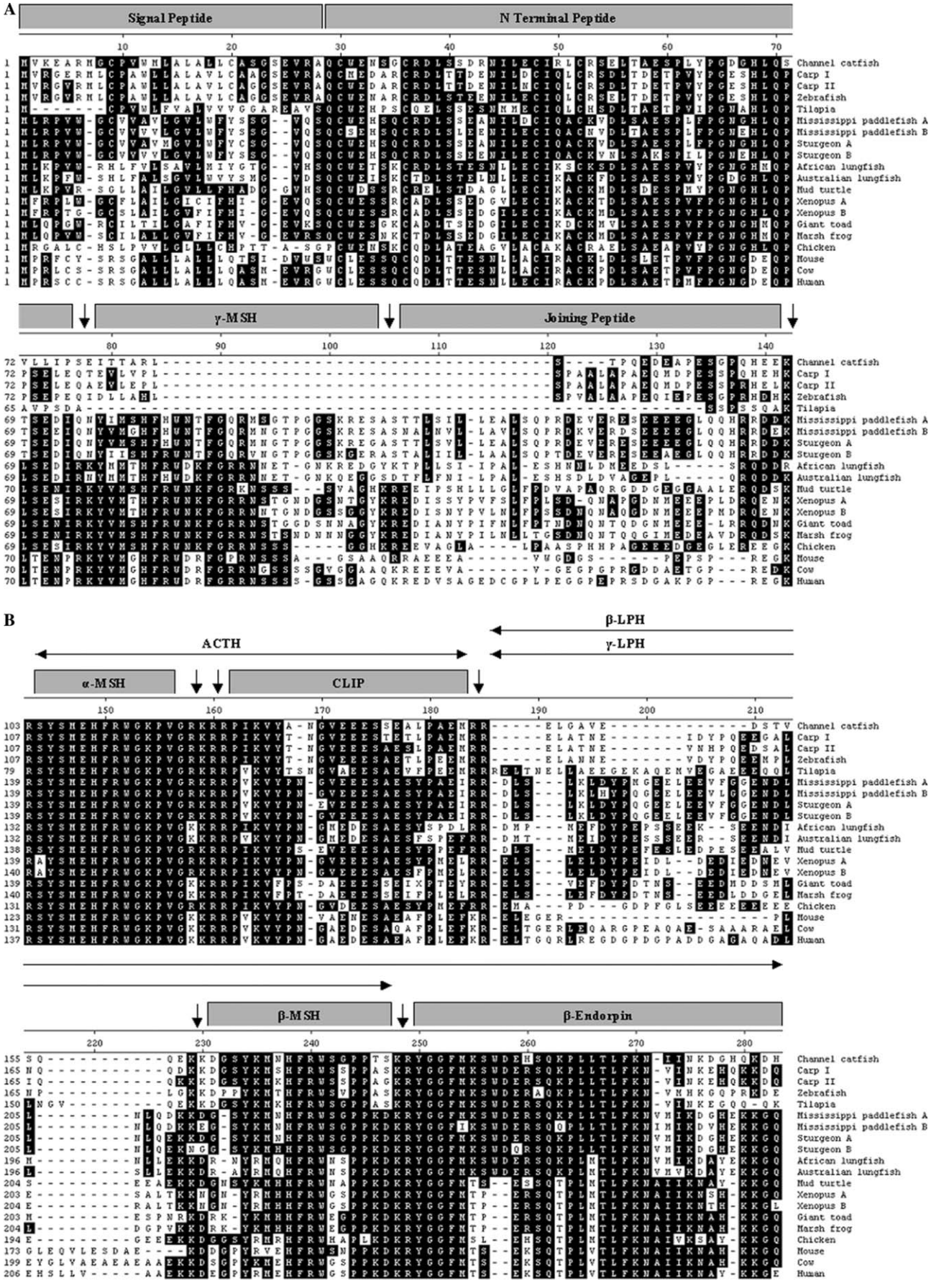
3.1. Cloning of the channel catfish POMC cDNA

Analysis of different cDNA sequences from pituitary and brain tissues revealed a single form of the *POMC* gene in channel catfish. The *POMC* cDNA is composed of 1164 bases including an open reading frame of 639 bases flanked by 123 bases of 5′ untranslated region (UTR) and 402 bases of 3′ UTR in which 4 potential poly(A)$^+$ signals, two AATAAA and two ATTAAA reside. Common AATAAA poly(A)$^+$ signals are 284 and 197 bases upstream of the poly(A)$^+$ sequences, while rare ATTAAA poly(A)$^+$ signals are much closer, 168 and 18 bases upstream of the poly(A)$^+$ sequences (Fig. 1). The sequence of the full-length cDNA is available in GenBank (Accession No. AY174050)

3.2. Structure of the catfish POMC protein

A multiple sequence alignment of the POMC precursors of several vertebrate species revealed the structure of catfish POMC precursor protein. It consists of 212 amino acids and encodes a signal peptide (SP, Met^1–Ala^{28}), N-terminal peptide (Gln^{29}–Glu^{101}), adrenocorticotropic hormone (ACTH, Ser^{104}–Met^{142}), α-melanocyte stimulating hormone (α-MSH, Ser^{104}–Val^{116}), corticotropin-like intermediate lobe peptide (CLIP, Arg^{121}–

Fig. 2. Multiple sequence alignment of POMC amino acid sequences of selected vertebrates. Identical amino acids are in white letters with black background. Designation of POMC derived peptides is written above the marked regions. Arrows show the potential cleavage sites. Accession numbers for POMC sequences are in Table 1.


A
Signal Peptide
N Terminal Peptide
γ-MSH
Joining Peptide
Channel catfish
Carp I
Carp II
Zebrafish
Tilapia
Mississippi paddlefish A
Mississippi paddlefish B
Sturgeon A
Sturgeon B
African lungfish
Australian lungfish
Mud turtle
Xenopus A
Xenopus B
Giant toad
Marsh frog
Chicken
Mouse
Cow
Human
B
β-LPH
ACTH
γ-LPH
α-MSH
CLIP
β-MSH
β-Endorpin

Met[142]), β-lipotropin (β-LPH, Glu[145]–His[212]), γ-lipotropin (γ-LPH, Glu[145]– Ser[177]), β-MSH (Asp[161]–Ser[177]), and β-endorphin (β-EP, Tyr[180]–His[212], Figs. 1 and 2). The channel catfish POMC protein does not contain γ-MSH, and most of the joining peptide and part of the γ-LPH are deleted (Fig. 2). Five dibasic cleavage sites formed by Arg and Lys amino acids are conserved. There are highly conserved regions in the active peptide regions. For example, in the N-terminal peptide region, four Cys[30,36,48,52], two Leu[39,56], two Pro[61,64], one Gly[65], and one Gln[70] residues are highly conserved. Similarly, at the N-terminal region of ACTH corresponding to α-MSH and at the C-terminal of γ-LPH corresponding to β-MSH, four amino acids (His–Phe–Arg–Trp) are always conserved among the species analyzed. In β-EP, highly conserved amino acid blocks such as Tyr[180]–Gly–Gly–Phe–Met[184] and Gln[192]–Lys–Pro–Leu–Leu–Thr–Leu–Phe–Lys–Asn[201] are visible while C-terminal of β-EP is more variable (Fig. 2). Catfish POMC precursor contains post-translational modification patterns including six protein kinase C phosphorylation sites (SdR[41–43], TaR[81–83], SyK[163–165], TsK[176–178], SkR[177–179], and SqK[191–193]), four casein kinase II phosphorylation sites (SgsE[22–25], TpqE[86–89], SqqE[155–158], and SwdE[186–189]), and one amidation site (vGRK[116–119]).

3.3. Phylogenetic analysis

The homologies of the various POMC precursors were identified (Fig. 3) and similarity of catfish POMC with other species is summarized in Table 1. The highest similarities were observed with the carp (*C. carpio*) POMC I (66.5%) and POMC II (67%) and zebrafish (*D. rerio*) POMC (64.2%), while the sea lamprey (*P. arinus*) proopiocortin (POC, 17.9%) and proopiomelanotropin (POM, 18.8%) genes were the most divergent. Chondrichthyan stingray (*D. akajei*) and dogfish (*S. acanthias*) showed low similarity, 38.2 and 43.4%, respectively. The average similarity was 46.95% and sarcopterygian African lungfish (*P. annectens*) and Australian lungfish (*N. forsteri*) showed slightly higher similarities than average, 48.1 and 49.1%, respectively. Chondrostean paddlefish (*P. spathula*) and sturgeon (*A. transmontanus*) POMCs were between 52.8 and 55.7%, which were higher than sarcopterygians but less than teleosts. Homology of mammalian species was similar to those of chondrichthyan fish (Table 1). Homology dramatically increases when only biologically active regions are used in similarity analyses (Shen et al., 2003). It is clear from the multiple alignments that the most highly conserved active peptide is α-MSH followed by β-MSH, β-EP, and CLIP while γ-MSH, JP, and the N-terminal of γ-LPH are the most divergent regions (Fig. 2).

3.4. Tissue expression of POMC gene

To determine *POMC* expression in different tissues, a 119 bp fragment (246–364) of catfish *POMC* gene including 16 bases from exon 2 and 103 bases from exon 3 was amplified. The real-time PCR analysis detected a high level of *POMC* mRNA expression in the pituitary while expression in the other tissues was very low comparatively (Fig. 4). Expression in anterior kidney was the highest among the non-pituitary tissues. Stomach and posterior kidney expression was slightly higher than in brain, gill, heart, liver, spleen, and muscle tissues where *POMC* expression was about at the same level. Skin expression was the lowest among all tissues analyzed (Fig. 4). Compared to the *POMC* mRNA levels in the pituitary, the concentration of *POMC* mRNA was

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
1		66.5	67.0	64.2	51.4	55.7	54.2	56.6	52.8	48.1	49.1	45.3	46.7	45.3	44.3	46.2	43.9	42.5	43.9	42.9	1	Channel catfish
2	39.4		91.4	80.6	55.3	52.7	51.4	51.8	49.5	48.4	48.4	44.1	46.4	46.8	41.9	44.1	43.7	42.3	44.6	42.3	2	Carp I
3	38.6	9.1		80.2	56.2	53.2	51.8	52.7	50.9	46.8	46.4	46.4	47.7	48.2	42.3	45.0	45.9	42.8	45.5	42.8	3	Carp II
4	43.4	22.5	23.1		52.9	52.7	52.3	52.7	52.3	49.1	48.2	44.6	44.6	45.5	41.4	44.1	43.2	39.6	41.4	40.5	4	Zebrafish
5	48.4	46.4	43.6	48.3		51.9	49.0	52.4	50.5	44.2	44.2	41.8	42.3	43.3	38.5	40.9	40.4	39.4	40.4	40.9	5	Tilapia
6	65.4	71.5	67.1	70.4	62.1		89.0	96.2	90.5	58.4	60.8	53.3	54.8	54.6	48.6	53.1	47.8	47.7	42.0	43.2	6	Mississippi paddlefish A
7	67.7	71.5	67.1	71.5	64.4	11.9		89.0	86.4	55.3	57.3	49.4	52.1	53.1	45.9	50.0	45.8	46.8	41.7	41.7	7	Mississippi paddlefish B
8	66.5	73.8	68.2	71.5	62.1	3.9	11.9		90.2	58.8	61.2	54.0	54.4	55.0	48.6	53.1	47.0	47.7	42.0	43.2	8	Sturgeon A
9	72.3	76.2	70.4	71.5	65.5	10.1	15.1	10.6		57.6	58.8	50.2	52.1	52.3	46.7	50.4	46.6	45.5	40.5	40.5	9	Sturgeon B
10	84.7	83.1	76.7	79.2	80.5	55.8	65.0	53.4	57.4		81.6	55.3	56.5	58.1	53.7	55.3	50.2	47.2	45.1	46.3	10	African lungfish
11	84.7	84.5	79.2	79.2	76.5	50.4	59.8	48.9	55.0	21.2		54.1	54.9	54.9	50.6	54.5	50.2	48.1	46.3	46.7	11	Australian lungfish
12	77.9	81.7	76.8	81.7	86.9	58.8	66.5	57.2	64.7	53.4	54.1		62.2	61.5	58.7	60.8	58.6	53.2	51.7	53.3	12	Mud turtle
13	76.6	75.5	69.6	79.2	78.5	57.8	64.6	58.7	63.7	60.1	60.1	43.7		91.1	70.3	71.0	57.8	51.5	49.4	51.7	13	Xenopus A
14	76.6	77.3	71.4	78.6	77.1	60.0	65.1	59.1	63.3	60.6	58.9	45.5	8.2		71.0	74.6	55.8	51.5	49.6	51.2	14	Xenopus B
15	81.9	87.9	82.5	89.3	82.7	70.4	78.3	70.4	75.3	64.5	70.1	50.4	34.5	34.3		83.8	52.2	51.1	46.7	49.4	15	Giant toad
16	79.2	83.7	78.6	83.7	78.5	63.3	71.4	63.3	68.7	59.8	59.8	48.4	34.7	31.0	16.4		52.2	52.8	49.2	50.4	16	Marsh frog
17	83.9	89.2	83.7	89.2	87.1	74.6	80.9	75.7	79.9	70.4	66.5	51.5	54.6	59.3	67.2	64.5		51.9	51.4	52.6	17	Chicken
18	84.2	77.8	76.5	83.4	74.1	68.0	72.3	69.1	76.9	70.5	67.2	62.5	60.4	60.9	60.8	57.1	66.4		77.9	75.7	18	Mouse
19	87.4	86.4	83.7	94.9	81.2	80.4	84.8	79.3	85.9	75.6	72.5	69.8	66.0	64.6	75.1	66.5	70.5	18.9		77.7	19	Cow
20	87.4	89.8	88.4	94.1	81.9	80.9	85.2	79.9	86.3	75.7	74.6	69.6	69.0	67.7	82.5	73.3	71.1	23.3	18.3		20	Human
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		

Fig. 3. Sequence divergence and similarities among different species. Percent divergence is calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign. Percent similarity compares sequences directly, without accounting for phylogenetic relationships.

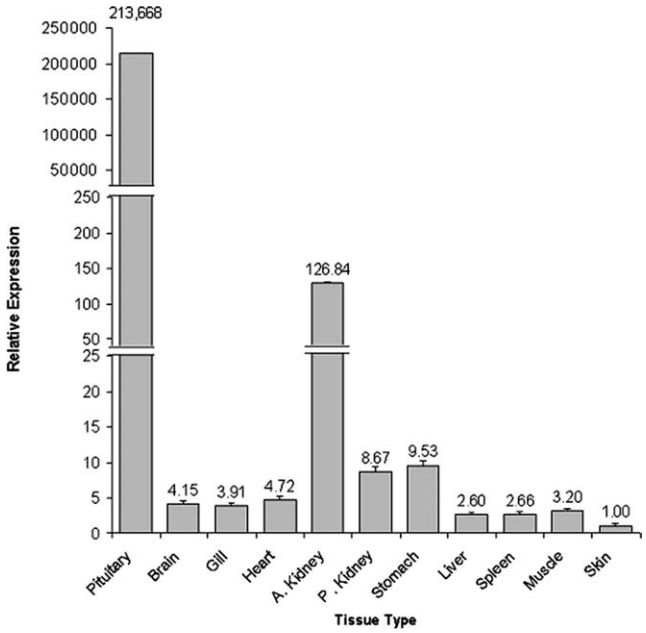


Fig. 4. Relative expression of *POMC* mRNA detected by real-time PCR analysis in channel catfish (*I. punctatus*) tissues. Expression levels of selected tissues are represented relative to the skin.

0.0594% in the anterior kidney, 0.0012–0.0045% in the other tissues, and 0.0005% in the skin.

4. Discussion

In the present study, we have cloned a single expressed form of proopiomelanocortin (*POMC*) from the pituitary and brain tissues of the channel catfish (*I. punctatus*). A single form of transcript has been found in tilapia (*Oreochromis mossambicus*, Lee et al., 1999), European sea bass (*D. labrax*, Varsamos et al., 2003), and zebrafish (*D. rerio*, Hansen et al., 2003). However, flounder (*Verasper moseri*), carp (*C. carpio*), salmon (*O. keta*), halibut (*Paralichthys olivaceus*), paddlefish (*P. spathula*), sturgeon (*A. transmontanus*), and trout (*O. mykiss*) contain two non-allelic *POMC* genes. The 3′ UTR of catfish *POMC* includes more than one poly(A)$^+$ signal, which has been the case in several other fish species such as gar (*L. osseus*), stingray (*D. akajei*), lungfish (*P. annectens* and *N. forsteri*), and paddlefish. Alternative polyadenylation of mRNA transcripts is not a rare event and about one-fifth of mRNAs undergo tissue or time specific alternative polyadenylation, which may affect stability and localization of mRNA (Beaudoing et al., 2000; Edwalds-Gilbert et al., 1997). Although alternatively polyadenylated *POMC* transcripts have mostly been reported from non-pituitary tissues recent studies showed the presence of alternatively spliced *POMC* mRNAs in the pituitary tissue (Lee et al., 1999; Shen et al., 2003). Poly(A)$^+$ signals in catfish *POMC* may yield differentially spliced mRNA transcripts but we did not observe *POMC* transcripts with different length 3′ UTRs. In catfish pituitary tissue, it is likely that the closest hexanucleotide ATTAAA signal is actually used as the poly(A)$^+$ signal since the majority of the poly(A)$^+$ signals are usually located about 11–23 bases upstream of the poly(A)$^+$ start site.

POMC mRNA encodes a 212 amino acid precursor containing five dibasic residues also found in other teleost POMCs. Amino acid numbers change in different classes and catfish has one of the shortest POMC proteins, which is slightly longer than tilapia, flounder A, and halibut II POMCs. Generally teleost POMCs are shorter than sarcopterygian fish and chondrosteans while chondrichthyan stingray and dogfish (*S. acanthias*) have the largest POMCs among the species analyzed (Table 1). Size differences are mainly due to absence or presence of melanocyte stimulating hormone (MSH) regions, amino acid insertions in the N-terminal protein, and amino acid extensions at the C-terminal of POMC precursor. Teleost fish do not contain γ-MSH and δ-MSH while sarcopterygians and chondrosteans have a γ-MSH region as in tetrapods. Similarly, chondrichthyan fish contain a new δ-MSH along with the γ-MSH region, which makes their POMCs largest among analyzed POMCs. Teleost fish POMCs contain five dibasic cleavage sites and organization of the functional peptides of channel catfish POMC is the same as other teleosts indicating similar proteolytic cleavage mechanisms. A close analysis of the cleavage sites revealed that Arg at the second position is more conserved than Lys or Arg at the first position. These dibasic cleavage sites support the inference that catfish POMC precursor can be processed to produce adrenocorticotropic hormone (ACTH), α-MSH, β-MSH, γ-lipotropin (γ-LPH), and β-endorphin (β-EP). Four highly conserved cysteine residues at the N-terminal peptide form two disulfide bridges and thus a hairpin loop structure, which contribute to the stability of the tertiary structure and sorting POMC to the regulated secretory pathway (Cool et al., 1995; Denef et al., 2001). Four highly conserved amino acids at the α-MSH region (His^{109}–Phe–Arg–Trp^{112}) and β-MSH region (His^{168}–Phe–Arg–Trp^{171}) are the core MSH sequences. Met-enkepalin core sequences, Tyr^{180}–Gly–Gly–Phe–Met^{184}, at the N-terminal of the β-EP are identical in all species analyzed except in Mississippi paddlefish, which has an Ile at fifth position (Fig. 2). Each biologically active region contains modifications between teleosts and others and subtle differences between catfish and other teleosts. Sea lamprey (*P. marinus*) proopiocortin (POC) has several amino acid insertions in the N-terminal peptide region. Amino acid insertions in γ-LPH are unique to some fish species such as salmon POMC II, trout POMC B, sea lamprey POC, and chondrichthyan dogfish and stingray. Similarly, only trout POMC A contains a unique 25 amino acid

C-terminal extension, which generates novel decapeptides (Salbert et al., 1992; Tollemer et al., 1997).

POMC protein sequences, which contain both conserved hormone regions and the highly variable spacer regions, were used to determine homologies between different species. As expected, homology was high in the species of same class and catfish were more similar to teleosts than other classes (Table 1 and Fig. 3). The close evolutionary relationship of channel catfish to cyprinids has been shown by single peptide alignments and mitochondrial genome analysis (Liu et al., 2001; Small and Nonneman, 2001; Waldbieser et al., 2003). Cyprinids are ancestrally tetraploid; a common ancestor underwent a genome duplication event. As a result, many cyprinid genes remain duplicated as it is observed in POMC gene. However, catfish, a member of order Siluriformes, is a diploid organism and does not share a common ancestor with the cyprinid fishes (order Cypriniformes, Nelson, 1994). Being evolutionarily close species with cyprinids especially with the model organism zebrafish would be beneficial to catfish in comparative genomic studies. The most conserved region of POMC precursor is α-MSH in all vertebrates, which may indicate its essential multifunctional role in physiological events including pigmentation, feeding behavior, and energy balance. Recently α-MSH has been linked with obesity (for review see Wardlaw, 2001).

The current research is the most complete survey reporting the multiple tissue expression of *POMC* gene in fish. As anticipated, the concentration of *POMC* mRNA expression in pituitary was much higher (1685 to 213,668-fold) than in non-pituitary tissues. High-level expression of *POMC* in pituitary could be attributed to its importance and involvement in important physiological processes including stress response (for review see Wendelaar Bonga, 1997). We found relatively high levels of *POMC* mRNA expression in the anterior kidney, which is the functional counterpart of mammalian bone marrow. Therefore high-level expression of *POMC* in anterior kidney could be attributed to its high importance and involvement in immune function. This is consistent with previous research describing the presence of *POMC* in the immune system and its potential involvement in immune responses in vertebrates (for review see Blalock, 1999). Presence of immunoreactive ACTH has previously been demonstrated in channel catfish leukocytes (Arnold and Rice, 2000). Non-pituitary tissues expressed *POMC* at lower levels, which may indicate local response mechanisms in those tissues. Channel catfish, an important aquacultured species, is exposed to various stressors in its culture environment and establishment of stress responses is important to permit efficient growth. The availability of the catfish *POMC* gene and protein sequence should provide molecular tools for understanding the role of POMC derived peptides in stress responses.

Acknowledgments

Special thanks goes to Mary Duke and Jimmie Warren for their technical assistance, to Dr. Lanie Bilodeau, Dr. Brian Bosworth, and Bethany Santucci for their constructive comments on the manuscript. Mention of a trade name, a proprietary product, or specific equipment does not constitute a guarantee or warranty by the US Department of Agriculture and does not imply approval to the exclusion of other products that may be suitable.

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